AD-A262 483

MENTATION PAGE

REPORT DATE	3 REPORT TYPE AND DATES COVERED
	FINAL 01 Jul 91 TO 30 Jun 92

4 TITLE AND SUBTITLE
WOUND HEALING AND CONNECTIVE TISSUE METABOLISM - THE ROLE OF HYPERBARIC OXYGEN THERAPY

5 FUNDING NUMBERS
AFOSR-91-0413
61102F
2312
A6

6. AUTHOR(S)
Dr Elvin Harper

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Univ of California, San Diego
Dept of Chemistry
9500 Gilman Drive
La Jolla, CA 92093-0506

8. PERFORMING ORGANIZATION REPORT NUMBER
AFOSR-TR- 93 0312

9. SPONSORING MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Dr Walter Kozumbo
AFOSR/NL
110 Duncan Avenue, Suite B115
Bolling AFB DC 20332-0001

10. SPONSORING MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION AVAILABILITY STATEMENT
Approved for public release;
distribution unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

Effect of hyperbaric oxygen on wound healing by growth promoting factors: Epidermal growth factor (EGF), platelet derived growth factor (PDGF) and transforming growth factor B (TGFB). These compounds are of particular interest since they have been reported to increase collagenase secretion. EGF when applied directly to skin increased the rate of healing by 100%.

14. SUBJECT TERMS			15. NUMBER OF PAGES
			16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19 SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRA
(U)	(U)	(U)	(UL)

NSN 7540-01-280-5500
Standard Form 298 (Rev 2-89
Prescribed by ANSI Std Z39-18
298-102

Elvin Harper, Ph.D.

91-0413

FINAL TECHNICAL REPORT

Wound Healing and Connective Tissue Metabolism - The Role of Hyperbaric Oxygen Therapy

Lieutenant Colonel George Wolf made a management visit to our laboratory on Monday, September 10, 1990. On the basis of our discussions of the progress made in our research endeavors we were encouraged to submit a proposal to continue our studies.

Specific Aim Number Four

Effect of hyperbaric oxygen on wound healing by growth promoting factors: Epidermal growth factor (EGF), platelet derived growth factor (PDGF) and transforming growth factor β (TGFβ). These compounds are of particular interest since they have been reported to increase collagenase secretion. EGF when applied directly to skin increased the rate of healing by 100%.

In order to accomplish this goal we carried out the following experiments.

We have produced an antibody directed against rabbit skin collagenase and utilized this protein in Western (protein) blotting to ascertain the presence or absence of the rabbit skin collagenase. Our data show that in tissue culture of rabbit skin the collagenase appears on day 3 through day 7. The Western (protein) blot is enhanced in the presence of epidermal growth factor (EGF) in a dose dependent manner at five and ten nanograms per ml of culture media. Thus by immunochemical criteria a stimulation of the rabbit skin collagenase was observed.

We have correlated this immunochemical data with enzymatic activity utilizing the ^{14}C glycine peptide release assay to quantitate the rabbit skin collagenase present. A rise in collagenase activity was obtained that parallels the stimulation induced by EGF found in the immunochemical Western (protein) blotting experiments. We now have in hand baseline data for normal and epidermal growth factor treated rabbit skin. In collaboration with Dr. Tom Neuman of our

institution we are beginning to examine the effect of a hyperbaric environment on these tissues. We then plan to conduct whole animal (rabbit) experiments.

We purified rabbit skin collagenase, prepared by a number of chromatography steps: sulfopropyl Sephadex, Sephacyl S-200, collagen Sepharose, and heparin Sepharose. The purified enzyme had a molecular weight of approximately 49,000 and an isoelectric pH of 5.0. One band on SDS-PAGE established homogeneity of the sample. This procedure was similar to one published previously in our laboratory (Bicsak and Harper, 1984).

Purified collagenase was then used as antigen for preparing polyclonal antibodies to collagenase in sheep. We have given multiple subcutaneous injections of collagenase to the animal, after first obtaining preimmune serum. The sheep continued to produce antibodies to collagenase 18 mos after the initial injection of antigen. This antiserum has been shown to react with collagenase in double immunodiffusion assays and/or protein blots (Harper et al. 1990). Dilutions of 1:200 routinely gave good precipitation of rabbit skin, collagenase on protein blots. Characterization of collagenase was carried out using this antiserum. We have demonstrated that collagenase cross-reacts with this antibody in a double immunodiffusion assay. Protein blots of collagenase identified one major species (M_r = 45,000) and a minor species (M_r = 50,000) of immunoreactive protein.

We prepared antiserum to purified rabbit skin collagenase. The antigen, rabbit skin collagenase (80-100 μg) was mixed 1:1 with complete Freund's adjuvant and injected subcutaneously into a sheep. Sera were examined on double immunodiffusion plates and immunoblots. We have given multiple subcutaneous injections of collagenase to the animal after first obtaining preimmune serum. The sheep continued to produce antibodies to collagenase 18 mos after the initial injection of antigen.

Collagenase Assay: Collagenase activity was measured in the ^{14}C-glycine peptide release assay (Harper et al. 1988). This assay uses neutral salt-soluble guinea pig skin collagen, purified after *in vivo* labeling with ^{14}C-(U)-glycine (Miller and Rhodes 1982). A solution of radioactive-labelled collagen was allowed to form fibrils prior to the addition of enzyme solution. Solutions to

be assayed were added to this fibrillar substrate, mixed, and the reaction allowed to proceed at 37°C until cleavage of substrate was detectable. The reaction was terminated by centrifugation in a Beckman 152 microfuge for 5 min at room temperature. Aliquots of supernatant were counted on a liquid scintillation counter. A control with trypsin alone was included to correct for counts released by any denatured or noncollagenous protein cleavage, and collagenase activity expressed as μg collagen degraded per min at 37°C

Activation of Procollagenase: Cultures that showed inhibition or lack of enzyme activity were activated by treatment with trypsin)Bauer et al. 1975) or aminophenyl mercuric acetate (APMA) (Sellers and Reynolds 1977). TPCK-trypsin (0.1 to 10 μg) was added to the medium and incubated for 10-30 min at room temp (20-22°C). A 5-10 fold excess of soybean trypsin inhibitor was used to stop the reaction, and the sample was assayed for collagenase activity as described above. Organomercurials also activate collagenase medium if incubated with 0.5-1.0 nM 4-aminophenyl mercuric acetate for 6-8 hrs at 25°C, and dialyzed at 4°C against 50 mM Tris-HCl, pH 7.4, 10 mM $CaCl_2$, 1 M NaCl (Sellers et al. 1977) prior to assaying for collagenase activity. If inhibitor was present, it was titrated out with a known amount of enzyme prior to attempting the activation step (Herron et al. 1986). Active sample were also treated with trypsin or APMA to determine the total possible collagenase activity present.

Collagenase Inhibitor Assay; Collagenase inhibition assays were conducted as above, except that inhibitor was incubated with collagenase for 20 min at room temperature prior to assaying the solution for enzyme activity. One unit of inhibitor inhibits one unit of collagenase. The mw of purified collagenase inhibitors isolated to date is approximately 25,000 daltons. If one assumes a 1:1 stoichiometry of enzyme to inhibitor on a molar basis (Cawston et al. 1981), then we calculated that 1 μg of enzyme (mw - 50,000 daltons) would be completely inhibited by 0.5 μg of inhibitor.

Protein (Western) Blotting: Cultures were examined for reactivity with sheep antiserum to rabbit skin collagenase prepared in our laboratory. Enzymes were electrophoresced on an SDS-polyacrylamide gel slab according to the procedure of Laemmli (1970). Proteins were transferred

onto nitrocellulose membranes (0.45 u) in a Trans-blot apparatus (Bio-Rad) at room temp in 8.4 mM Tris, 192 mM glycine, 20% methanol. Membranes were incubated for 2 h to overnight with sheep antiserum to rabbit skin collagenase (1:200) dilution. Alkaline phosphatase conjugates were then added, followed by substrates nitro blue tetrazolium (NB) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) at pH 9.8. Development of the membrane was stopped with distilled water. Prestained SDS-PAGE standards and biotinylated standards were included in each experiment to determine protein transfer efficiency and to estimate molecular wts of the immunoreactive species, respectively.

Rabbit Skin Cultures: Samples were obtained from normal rabbits. Tissues were surgically removed and placed in Dulbecco's modified Eagle's medium supplemented with penicillin, streptomycin and fungazone. The culture vessels were incubated at 37°C under a humidified atmosphere of 95% air, 5% CO_2. The media are collected every day and assayed for collagenolytic activity and inhibitory capacity.

References

Bauer, E.A., Stricklin, G., Jeffrey, J., Eisen, A. *Biochem. Biophys. Res. Comm.* 64:2322-40, 1975.

Bicsak, T., and Harper, E. *J. Biol. Chem.* 259(21):13145-13150, 1984.

Cawston, T. E., Galloway, W. A., Mercer, E., Murphy, G., Reynolds, J. *Biochem. J.* 195:159-165, 1981.

Harper, J., Amiel, D., and Harper, E. *Connect Tissue Res.* 17:253-259, 1988.

Harper, J., Amiel, D., and Harper, E. *Orth. Res. Soc.* 15(1):535, 1990.

Herron, G. S., Banda, M. J., Clark, E. J., Gavrilovic, J., and Werb, Z. *J. Biol. Chem.* 261:2814-18, 1986.

Laemmli, M. K. *Nature* 227:680-685, 1970.

Miller, E. J. and Rhodes, R. K. *Methods Enzymol.* 82:33-64, 1982.

Sellers, A., Cartwright, E. C., Murphy, G., and Reynolds, J. J. *Biochem. J.* 163:303-307, 1977.

Sellers, A. and Reynolds, J. J. *Biochem. J.* 167:353-360, 1977.